Menopause is associated with decreased postprandial ghrelin, while a history of anorexia nervosa is associated with increased total ghrelin

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Abstract: INTRODUCTION: Middle age has been linked with various dysfunctional eating patterns in women. The hormone ghrelin is related to food intake, with plasma levels rising before eating and decreasing immediately afterwards. Animal research has shown that estradiol is an antagonist of ghrelin. Given that both menopause and anorexia nervosa (AN) are states characterised by reduced estradiol, the goal of the present study was to investigate for the first time whether menopausal status and a history of AN are linked with altered ghrelin levels in middle-aged women. Based on previous research, we hypothesised that a) post-menopausal women would demonstrate comparably increased ghrelin after food intake and b) women with a history of AN would exhibit increased total ghrelin levels. METHODS: Healthy, middle-aged women (N=57) were recruited. N=31 were post-menopausal and n=27 had a history of AN. Plasma was repeatedly collected before and after a meal standardised in terms of caloric content. Areas under the curves were calculated to indicate total (AUCg) and postprandial ghrelin (AUCi). RESULTS: Menopausal status was linked with postprandial ghrelin (AUCi -1.6±2.2 vs. -2.9±2.6; p=.058), while a history of AN was linked with total ghrelin (AUCg 36.2±5.6 vs. 39.0±3.7; p=.050). There were no interaction effects (both p>.466). A closer examination of the effects revealed that post-menopausal women showed marginally greater decreases in ghrelin immediately after food intake (p=.064) and marginally greater re-increases after 60 min (p=.084) when compared to pre-menopausal women. Women with a history of AN had significantly higher total ghrelin when compared to women without a history of AN (p=.042). DISCUSSION: Post-menopause was linked with higher sensitivity of ghrelin to food intake (trend), while a history of AN was related to greater total ghrelin. Future research should investigate to what extent the observed alterations in ghrelin may affect dysfunctional eating behaviour during middle age.

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Short tile: Menopause and postprandial ghrelin

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Abstract

Introduction

Middle age has been linked with various dysfunctional eating patterns in women. The hormone ghrelin is related to food intake, with plasma levels rising before eating and decreasing immediately afterwards. Animal research has shown that estradiol is an antagonist of ghrelin. Given that both menopause and anorexia nervosa (AN) are states characterised by reduced estradiol, the goal of the present study was to investigate for the first time whether menopausal status and a history of AN are linked with altered ghrelin levels in middle-aged women. Based on previous research, we hypothesised that a) post-menopausal women would demonstrate comparably increased ghrelin after food intake and b) women with a history of AN would exhibit increased total ghrelin levels.

Methods

Healthy, middle-aged women (N=57) were recruited. N=31 were post-menopausal and n=27 had a history of AN. Plasma was repeatedly collected before and after a meal standardised in terms of caloric content. Areas under the curves were calculated to indicate total (AUCg) and postprandial ghrelin (AUCi).

Results

Menopausal status was linked with postprandial ghrelin (AUCi -1.6±2.2 vs. -2.9±2.6; p=.058), while a history of AN was linked with total ghrelin (AUCg 36.2±5.6 vs. 39.0±3.7; p=.050). There were no interaction effects (both p>.466). A closer examination of the effects revealed that post-menopausal women showed marginally greater decreases in ghrelin immediately after food intake (p=.064) and marginally greater re-increases after 60 min (p=.084) when compared to pre-menopausal women. Women with a history of AN had significantly higher total ghrelin when compared to women without a history of AN (p=.042).

Discussion

Post-menopause was linked with higher sensitivity of ghrelin to food intake (trend), while a history of AN was related to greater total ghrelin. Future research should investigate to what extent the observed alterations in ghrelin may affect dysfunctional eating behaviour during middle age.

Introduction

Middle age has repeatedly been found to be linked with dysfunctional eating patterns in women. A recent survey showed that 6-16% of women between 40 and 66 report clinically relevant levels of eating concerns, restrained eating, weight concerns, and body shape concerns (1). The hormone ghrelin has been demonstrated to be related to food intake (e.g., 2), with plasma levels rising before eating and decreasing immediately afterwards (3, 4). In female animals, ghrelin appears to promote food intake, and, interestingly, to a greater extent in ovariectomised rats when compared to intact or estradiol (E2) treated ovariectomised rats (5, 6). This suggests that E2 antagonises the orexigenic effects of ghrelin in animals.

Based on these findings, it is conceivable that in humans, the decline in E2 as occurring during menopause may lead to elevated levels of ghrelin, which in turn may promote dysfunctional eating behaviours (e.g., binge eating). However, the literature comparing ghrelin in pre- versus post-menopausal women is highly contradictory, with some studies indeed observing comparably higher levels during the peri-menopause (7, 8), while others find attenuated levels in post-menopausal women (9), and yet others report no changes (10-12). Notably, all of these studies investigated the effects of menopausal status on unstimulated (fasting) ghrelin levels, while none have so far examined stimulated ghrelin in response to food intake in pre- versus post-menopausal women (i.e., postprandial ghrelin).

Interestingly, underweight patients with anorexia nervosa (AN), a disorder characterised by restrained eating, have also been shown to exhibit higher levels of plasma ghrelin when compared to normal weight individuals (13). This apparently paradoxical finding may be explained by the fact that in humans, ghrelin is one of the neural signals involved in foraging for food, a mechanism which appears to involve the neuropeptide Agouti-related Protein localised in the arcuate nucleus (14-17, but see also 18). While foraging for food may be offset in obesity as a result of abundantly available food (19), it has suggested to be related to reduced food intake as present in AN (20). Interestingly, the elevated plasma ghrelin concentrations in patients with AN appear to normalise after treatment. However, studies with long-term follow-ups are lacking and in light of the above evidence for E2-dependent changes in ghrelin, the question arises of whether this healthy state persists into middle age, or whether recovered women with a history of AN may be at risk of re-developing dysfunctional patterns of eating behaviour during middle age.

In sum, the currently available evidence suggests that dysfunctional eating patterns in middle-aged women may be related to altered ghrelin levels, which in turn may be traced back to declines in E2 as present in post-menopausal women and in women with a history of AN. The goal of this study was to examine for the first time whether ghrelin responses to a standardised meal differ between pre- versus post-menopausal women. In addition, we aimed to explore whether a history of AN would be linked with altered ghrelin levels in middle age. Based on the above literature, we hypothesised that a) post-menopausal women would demonstrate higher ghrelin after food intake and b) women with a history of AN would have higher total ghrelin when compared to those with no history of AN, and c) that these effects would be additive rather than interactive.

Methods

Participants

Women were recruited via e-mail, newspaper articles, flyers, and poster advertisements. Exclusion criteria for all participants were: smoking more than five cigarettes per day, alcohol consumption of more than three standard units per day, intake of medication, being over- or underweight (BMI > 25 or < 18.5), pregnancy, irregular menstruation cycle, hysterectomy, ovariectomy, or hormonal substitution, vegetarian or vegan diet, food allergies, and being a professional athlete. Furthermore, all women with self-reported mental or physical illnesses were excluded. Fifty-seven healthy, middle-aged women took part in the study (n=7 smokers). The women were divided into four groups: pre-menopausal without a history of AN (n=15), pre-menopausal with a history of AN (n=11), post-menopausal without a history of AN (n=15), and post-menopausal with a history of AN (n=16). All pre-menopausal women reported a regular menstrual cycle and did not use hormonal contraceptives. In accordance with the definition of menopause (21), women were classified as post-menopausal if they reported the absence of a menstrual cycle for twelve months or more.

Protocol

Potential participants were contacted by telephone and screened for eligibility. All were then asked about their pre- or post-menopausal status. A history of AN occurring between the ages of 12 and 25 was assessed by means of the structured clinical interview for DSM IV (SCID; 22). This time frame was chosen since AN

most typically begins during adolescence (e.g., 23). Current absence of an AN diagnosis was confirmed using the SCID. In addition, the SCID was used to confirm absence of comorbidity with other mental disorders. All SCIDs were conducted by a clinical psychologist (SS). In pre-menopausal women, plasma levels of E2 were to be determined two days before ovulation. To this end, all women received an ovulation testing package, which included the Clearblue© digital ovulation test (Swiss Precision Diagnostics GmbH, Geneva, Switzerland) and instructions on how to determine the precise date of their ovulation (24). The date of the individual appointment was determined by adding a full menstrual cycle to the ovulation date as identified by the participants.

The laboratory appointment began at 0800 h. Participants had been instructed to adhere to a fasting period of at least eight hours before the appointment. Subsequently, height, weight, waist and hip measurements were taken, a registered nurse inserted a vein catheter, and the first blood sample (blood sample 1: 0 min) and a saliva sample were taken. Participants filled in visual analogue scales (VAS 1) to record their subjective feelings of hunger (25). Next, a standard breakfast according to the Swiss Nutrition Report (26) was served, consisting of bread, butter, jam, yogurt, and a slice of apple and orange. The meal contained 10.9% proteins (35.5 kcal), 20.3% fat (66.2 kcal), and 67.9% carbohydrates (221.4 kcal), with a total of 323.1 kcal, which is approximately 20% of the daily energy expenditure of a woman with a normal BMI (27). After the meal, five more blood samples were taken (blood sample 2: +20 min; blood sample 3: +40 min, blood sample 4: +60 min, blood sample 5: +120 min, blood sample 6: +180 min). Blood samples were used to determine acylated ghrelin in plasma (gh_0, gh_20, gh_40, gh_60, gh_120, gh_180) while the saliva sample was taken to assay E2. Visual analogue scales were filled in at baseline (VAS1) and after each blood sample (VAS 2-6). Towards the end of the appointment, a standardised clinical interview based on DSM-IV-TR criteria was conducted with all women who had reported a history of AN to verify their former diagnosis (28).

Blood sampling and analysis of ghrelin

Blood samples were collected into EDTA containing Monovette tubes. Samples were immediately pipetted into Eppendorf tubes containing AEBSF (4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride) [2 mg/mL blood] in order to prevent the degradation of acylated ghrelin. Blood samples were centrifuged for

10 min at 4°C and 3500 rpm, and the plasma was immediately pipetted into Eppendorf tubes and stored at -80°C until analysis. Plasma concentrations of human acylated ghrelin were determined using a sandwich immunoassay (MSD® Human Active Ghrelin Assay; Meso Scale Discovery, Gaithersburg, USA). The assay was conducted as per the manufacturer's instructions, with the exception that 50 µL instead of 25 µL of sample volume were used to increase sensitivity. Quality control samples were placed at the beginning and end of each plate. The intra- and inter-assay coefficients of variation were 6.0% and 8.2%, respectively.

Saliva sampling and analysis of estradiol

Saliva samples for E2 determination were collected with ultrapure polypropylene tubes (SaliCaps, IBL, Hamburg, Germany). Participants were asked to release 2.5 ml saliva into the SaliCap with the help of a polypropylene straw. The saliva samples were stored at -20°C until analysis. The biochemical analyses were conducted using a standard luminescence immunoassay in our laboratory (IBL, Hamburg, Germany). Intra- and inter-assay coefficients were below 10%.

Hunger

Subjective feelings of hunger were assessed using a 10 cm VAS (25) and using the end points "hungry" versus "full". Each VAS (1-6) was presented immediately before the respective blood sample was taken.

Statistical analysis

All data were checked for normal distribution using the Kolmogorov–Smirnov test. To allow for parametric testing, all biological variables were log-transformed. The four groups were then compared in terms of Body Mass Index (BMI), the waist-to-hip (WHR) ratio, E2, and baseline levels of ghrelin and hunger, using univariate ANOVAs with Gabriel's post-hoc test, and the Kruskal-Wallis test.

First, areas under the curve with respect to ground (AUCg) and increase (AUCi) were calculated according to the formulae outlined by Pruessner, Kirschbaum (29). This method is frequently used in neuroendocrinological research, and particularly when response curves to a specific stimulus are being studied. The approach allows to a) capture *total endocrine output* in response to a stimulus (i.e., including

baseline levels of the respective hormone), represented by the AUCg, and b) *endocrine responses* to the standardised meal (i.e., excluding baseline levels of the respective hormone), represented by the AUCi (see e.g. 30 for an illustration of the AUCg and AUCi). Univariate ANOVAs were then conducted to compare the two groups in terms of both AUCs. Finally, in cases of significant group differences, postprandial changes in ghrelin were explored by means of growth curve models including both linear and quadratic time effects, group effects, and time by group effects were calculated.

Because ratings of hunger (VAS 1-6) were not normally distributed, the nonparametric Friedman's ANOVA was used to test possible time effects in response to the meal, and the Kruskal-Wallis test was used to test for group differences. Spearman's rank correlations were calculated to test for associations between the hunger and ghrelin AUCg and AUCi, respectively.

All analyses were two-tailed, with the level of significance set at $p<.05$. All calculations were performed using SPSS (Chicago, Illinois, USA) and data are presented as means ± standard errors.

Ethical approval

The study protocol was reviewed by the Research Ethics Committee of the University of Zurich, Switzerland and written informed consent was obtained from all participants.

Results

Participant characteristics

Descriptive data and baseline levels of ghrelin, E2 and the VAS according to group (N=57 women, aged from 40 to 60 years) are displayed in Table 1. While post-hoc testing revealed an expected difference in E2 levels between pre- and post-menopausal women irrespective of AN status (all $p<.005$), the four groups did not differ in terms of BMI and WHR.

Participants with a history of AN reported that, on average, they had been diagnosed with AN 32 years ago (28 years for pre-menopausal women, 35 years for post-menopausal women) and that they had had only one anorexic episode. In around two thirds (65%) of the sample, this episode occurred between 12 and

18 years of age and in around one third (35%) occurred between 19 and 25 years. The mean duration of this episode was four years, and the average number of years since recovery was 28 (range: 15-38).

-Insert Table 1 here-

Postprandial changes in ghrelin: main group effects and interaction effects

Univariate ANOVAs including the two factors menopausal status and a history of AN yielded no effect of menopausal status on the AUCg (pre-menopausal 37.3±5.4 vs. post-menopausal 37.8±4.6; $F(1, 51)=0.033$, $p=.856$, $\eta^2=.001$), while a trend emerged in terms of the AUCi (pre-menopausal -1.6±2.2 vs. post-menopausal -2.9±2.6; $F(1, 51)=3.770$, $p=.058$, $\eta^2=.069$). By contrast, a history of AN was related to the AUCg (without history 36.2±5.6 vs. with history 39.0±3.7; $F(1, 51)=4.019$, $p=.050$, $\eta^2=.073$), while no effect on the AUCi became apparent (without history -2.2±3.0 vs. with history -2.5±1.9; $F(1, 51)=0.092$, $p=.763$, $\eta^2=.002$). Neither of the interaction terms was significant (AUCg: $F(1, 51)=0.537$, $p=.467$, $\eta^2=.010$; AUCi: $F(1, 51)=0.233$, $p=.631$, $\eta^2=.005$).

Postprandial changes in ghrelin: role of menopausal status

Dividing women into pre-menopausal (n=26) and post-menopausal (n=31), univariate ANOVAs found there was no group difference in the AUCg ($F(1,46)=0.92$, $p=.343$, $\eta^2=.020$), while the AUCi was larger by trend in pre-menopausal women when compared to post-menopausal women, when controlling for a history of AN, smoking, and intake of alcohol ($F(1,45)=3.88$, $p=.055$, $\eta^2=.079$). In line with these findings, growth curve modelling revealed neither a main effect of time (linear: UC<-.01, $p=.722$; quadratic: UC<.01, $p=.332$) nor of group (UC=-0.17, $p=.276$) in terms of postprandial ghrelin levels, but trends in terms of the interactions between time and group (linear: UC<-.01, $p=.064$; quadratic: UC<.01, $p=.087$). These indicated marginally greater decreases and increases in ghrelin in pre- vs. post-menopausal women (see also Figure 1).

-Insert Figure 1 here-

Postprandial changes in ghrelin: role of a history of AN

Dividing women into those without a history of AN (n=30) and those with a history of AN (n=26), univariate ANOVAs found the AUCi did not differ between groups ($F(1,46)=0.60$, $p=.442$, $\eta^2=.013$), while the AUCg was increased in women with a history of AN when compared to women without a history of AN, when controlling for menopausal status, smoking, or intake of alcohol $F(1,45)=4.39$, $p=.042$, $\eta^2=.089$; see also Figure 2).

-Insert Figure 2 here-

Mirroring this finding, growth curve modelling revealed a significant main effect of time (linear: UC<-.01, $p=.026$; quadratic: UC<.01, $p=.009$) and a trend main effect of group regarding postprandial ghrelin levels (UC=-0.29, $p=.054$), indicating greater overall ghrelin levels in women with a history of AN when compared to those with no history of AN. By contrast, both time by group interaction terms remained insignificant (linear: UC<.01, $p=.970$; quadratic: UC<.01, $p=.942$).

Postprandial changes in hunger

Nonparametric ANOVA revealed a significant effect of time in terms of hunger ratings (VAS) in both pre-menopausal ($\chi^2(5)=78.0$, $p<.001$) and post-menopausal women ($\chi^2(5)=82.0$, $p<.001$). The mean rank of the AUCg appeared to be higher in post-menopausal women (mean rank=32.6) when compared to pre-menopausal women (mean rank=25.6), but again, this difference was not significant ($Z=-1.6$, $p=.114$). The AUCi did not differ between the two groups ($Z=-0.711$, $p=.477$). Neither the AUCg nor the AUCi differed between participants with or without a history of AN ($Z=-.085$, $p=.932$; $Z=-.333$, $p=.74$). There were significant correlations between the hunger AUCg and the ghrelin AUCg ($r=-0.37$, $p=.006$), and there was a trend in the correlation between the hunger AUCi and the ghrelin AUCi ($r=-0.26$, $p=.055$).

Discussion

We report two main findings. First, post-menopausal women showed more pronounced decreases and re-increases in postprandial ghrelin than pre-menopausal women (trend). Second, women with a history of AN

showed a greater total ghrelin output (AUCg) when compared to women without a history of AN. There was no interaction between menopausal status and a history of AN, suggesting independent effects on postprandial ghrelin.

This study appears to be the first to have investigated both unstimulated (fasting) and stimulated (postprandial) ghrelin levels across menopause. The finding that pre- and postmenopausal women did not differ in unstimulated ghrelin levels is in line with the majority of previous studies conducted on the subject (10-12). The finding of marginally greater postprandial decreases and re-increases during post-menopause is novel and suggests that these women are more responsive in terms of the effects of food intake on ghrelin secretion. In other words, the finding could indicate a stronger link between food intake and ghrelin as circulating concentrations of E2 decline during the menopausal transition (i.e., greater sensitivity), while unstimulated levels remain unaltered. However, these findings need to be interpreted with caution, since only trends were observed (p=.058, p=.064, p=.087). On the flip side, animal studies have demonstrated for E2 to exert an inhibitory effect on the effects of injected ghrelin on food intake (5, 6). Further research is thus needed to examine whether the possible loss of a suppressive effect of E2 on ghrelin could be associated with dysfunctional patterns of eating behaviour (e.g., binge eating) and weight gain as sometimes observed after menopause (31, 32). Notably, such studies would have to consider the role of BMI as done in the present study, since body weight is in turn strongly linked with changes in E2 during the menopausal transition (33).

While in general, the size of postprandial changes in ghrelin in our study is comparable to previous studies (34-37), we also found that women with a history of AN showed higher unstimulated (fasting) and stimulated (postprandial) ghrelin levels compared to women without a history of AN. Our finding seems to stand in contrast to prior research in remitted patients with AN, which has shown that ghrelin tended to decrease from elevated to normal concentrations as weight was being restored (13, 38, 39). However, these studies assessed ghrelin shortly after re-nutritional interventions, while the anorexic episodes in the present study had, on average, occurred over 30 years ago. Interestingly, ghrelin has been found to rise in response to pictures of food (40) and to have positive relations with restrained eating (41) in healthy volunteers. The increased ghrelin levels in our women with a history of AN might thus be paralleled by re-occurring cognitive and behavioural features of AN on a subclinical level and in absence of underweight (e.g., perseverative

thoughts about eating). However, further research is warranted to test these hypotheses, and to investigate the biological meaning of the observed alterations in individuals with a history of AN, especially given that we only found a small effect of this on ghrelin in the present study.

Our study presents with a number of strengths: It is the first to examine the impact of menopause on postprandial ghrelin, and the first to test whether women with a history of AN continue to show abnormalities in ghrelin production. Furthermore, we were careful in selecting healthy women to rule out any influences of confounders on our results (e.g., intake of medication). Limitations of our study include the relatively small sample size, and the fact that ovulation dates for pre-menopausal women were not confirmed by means of LH testing. Notably, all women had regular menstrual cycles, allowing for relatively precise calculations of ovulation dates. Further limitations are that we only measured acylated but not deacylated ghrelin, although the latter is also involved in the regulation of food intake, and that limited funds for this study prevented us from including a +10 min measurement time point, which corresponds to the half-life of acylated ghrelin. Also, due to the cross-sectional design of the study, it cannot be determined whether the observed alterations in postprandial ghrelin levels in post-menopausal women and in women with a history of AN affect food intake and are conducive to abnormal eating behaviour, or whether abnormal eating behaviour in these women affects ghrelin levels. Related to this, although all women were free of current AN according to the SCID, we did not assess subclinical cognitive or behavioural symptoms of AN, which could have re-occurred in absence of underweight. Longitudinal and mechanistic studies might provide further insight into the relationship between ghrelin levels and eating behaviour in middle-aged women.

In conclusion, the present study provides initial evidence for menopausal status to be related to ghrelin levels after food intake, such as that post-menopausal women appear to show a more sensitive response pattern. Further studies are now needed to test how ghrelin in turn affects food intake and – potentially – abnormal eating behaviour in pre- versus post-menopausal women. The finding of enhanced total ghrelin levels in middle-aged women with a history of AN indicates that the elevated levels may be linked to re-occurring cognitive and behavioural features of AN in absence of underweight – a hypothesis which should be tested by future research. Ideally, such studies would go beyond simply correlating hormonal levels with scores on eating behaviour questionnaires and explore the assumed associations as they unfold in

real life (e.g., by means of ambulatory assessments). Importantly, given the role of ghrelin in reward-based systems, cue-stimulated hedonic eating should be examined in addition to eating induced by hunger after food deprivation. Together, this kind of research could shed light on why the menopausal transition may represent a window of vulnerability for maladaptive changes in eating behaviour.

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Figure legends

Figure 1 Postprandial changes in ghrelin (pg/ml) in pre- (n=26) versus post-menopausal women (n=31). Data are presented as mean ± standard errors. Growth curve modelling revealed a trend in terms of the effect of menopausal status on postprandial decreases and increases in ghrelin.

Figure 2 Area under the curve with respect to the ground (AUCg) of postprandial ghrelin split by presence (n=27) vs. absence (n=30) of a history of anorexia nervosa (AN). Data are presented as mean ± standard errors. A univariate ANOVA revealed that women with a history of AN had significantly greater postprandial ghrelin secretion when compared to those without prior AN. *$p < .05$.